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13. ABSTRACT (Maximum 200 words) This report describes a method for the extended storage of human platelets. The platelet storage is performed at 4°C to eliminate the bacterial contamination problems associated with conventional storage. To abrogate platelet storage lesions which occur during the 4°C storage, the cells are treated with second messenger effectors which stabilize the cells by inhibiting the biochemical events which lead to storage lesions. Thus, following storage the cell remain active. Following 5 days of storage at 4°C, platelets treated with this stabilizing solution display activity profiles as follows: 92% recovery of cell number; 45% ADP-induced aggregation; 80% collagen-induced aggregation; 61% hypotonic shock response; 10.5% extent of shape change, 74% expression of the surface marker GPIb; and 44% expression of the activation marker GMP-140. After storage at 4°C for 9 days the treated platelets yielded the following functional activity values: 91% recovery of cell number; 43% ADP-induced aggregation; 72% collagen-induced aggregation; 48% hypotonic shock response; 11.6% extent of shape change; 64% expression of GPIb; and 50% expression of GMP-140. The results of this project demonstrate that the implementation of this method will allow platelets to be stored at 4°C for extended periods without loss of activity.

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Background

During the previous two years of this project, we generated data demonstrating that the biochemical stabilization of platelets by the application of specific second messenger effectors allows for the extended storage of platelets at 4°C. As detailed in LifeCell's 18 month and 2 year interim reports entitled "Prolonged Preservation of Human Platelets for Combat Casualty Care", a stabilization formulation, ThromboSol™, was developed which allowed platelets which had been stored at 4°C for up to 9 days to display good recovery of functional activity.

Briefly, these reports detail results which demonstrated the following:

- 1) The most effective combination of second messenger effectors and membrane stabilizers consist of amiloride (0.25mM), adenosine (0.1mM), sodium nitroprusside [SNP] (50uM), dipyridamole (40uM), quinacrine (0.2uM) and ticlopidine (0.75mM). This formulation is designated as ThromboSol in this report.
- 2) ThromboSol can be added directly to the platelet concentrate, which eliminates any compromising effects to the erythrocyte or plasma fractions of the procured whole blood unit.
- 3) The ThromboSol formulation can be prepared as a sonicated suspension in buffered saline which can be delivered via injection through a sterile port or by use of a sterilely docked satellite bag.
- 4) Electron microscopic analysis of platelets stored at 4°C, in the presence of ThromboSol, demonstrated that these cells were spherical in shape with good cytoplasmic organization, homogeneously distributed granules and intact plasma membranes.
- 5) ThromboSol-treated platelets stored at 4°C for 9 days displayed a 3-fold higher response to a low concentration (5uM) or high concentration (20uM) of ADP than conventionally stored platelets stored for 5 days.
- 6) ThromboSol-treated platelets stored at 4°C for 9 days yielded an equivalent response to collagen (20ug/ml) as conventionally stored platelets stored for 5 days.
- 7) Hypotonic shock response measurements demonstrated that the ThromboSol-treated platelets stored at 4°C for 9 days gave equivalent recovery to conventionally stored platelets at day 5.
- 8) FACS analysis to measure the expression of the activation marker GMP-140 (CD62) reveals that the conventional storage method results in platelet population with a high expression of this activation marker (54%), while only 32% of the ThromboSol-treated platelets stored at 4°C expressed this activation marker.

9) The use of ThromboSol for the cryopreservation of platelets allows for the reduction of the DMSO concentration threefold (2% final) as compared to the conventional cryopreservation methods approved by the AABB.

10) The platelets cryopreserved with ThromboSol and 2% DMSO yield a cell population, following thaw which displays higher recovery of cell number and functional activity than conventionally cryopreserved platelets.

This report documents the data generated during the final stage of this project. Outlined in this report is the analysis of the use of ThromboSol for the extended storage of platelets developed since the "Prolonged Preservation of Human Platelets for Combat Casualty Care" 2 year report submitted in April, 1995.

4°C Storage of Platelets with ThromboSol

New Assay System

In order to better analyze the recovery of platelet function following storage either under standard conditions or at 4°C with ThromboSol, a new assay was introduced to extend the number and scope of our in-vitro assay systems. The new technique is the extent of shape change assay (ESC). The ESC is an optical measurement of the discoid to spherical shape change that occurs as the initial activation event of platelets following stimulation with the agonist ADP. The assay is performed according to the protocol established by the BEST group. Briefly, the technique is performed as follows:

- 1) the platelet sample is incubated at 37°C for 30 minutes.
- 2) a 500 ul aliquot of PPP and PRP are placed in an aggregometer with constant stirring and measured for light transmission with water as a blank.
- 3) the PRP is then blanked against PPP and 20 ul of 0.1 M EDTA is added to the sample directly followed by 10 ul of 1 mM ADP.
- 4) the deflection from the baseline is measured at its peak and termed T_{ESC} .
- 5) the ESC is determined according the following formula:

$$\%ESC = \{ \log[(T_{PPP} - T_{PRP}) \times T_{ESC}] - \log T_{PRP} \} / \{ \log T_{PRP} - \log T_{PPP} \}$$

Table 1 displays the results of the measurement of ESC of PC (n value = 5) stored at 22°C or at 4°C in the presence or absence of ThromboSol.

Table 1

Extent of Shape Change Assay - ThromboSol-Treated Platelets vs. Control Platelets			
	Day 1	Day 5	Day 9
Control - 22°C	23.2 +/- 5.0	21.9 +/- 2.8	19.3 +/- 4.0
Control - 4°C	11.9 +/- 3.4	6.3 +/- 2.1	6.0 +/- 1.3
ThromboSol - 4°C	14.9 +/- 4.0	11.6 +/- 2.2	11.5 +/- 2.0

These results demonstrate that platelets stored at 4°C in the presence of ThromboSol yield a good recovery of ESC, indicating that these cells retain viable hemostatic properties. The ESC values are not as high as those seen with the control 22°C stored platelets. This is probably due to the fact that the ThromboSol-treated platelets already display a spherical morphology due to the 4°C storage and thus are not beginning the ESC assay as discoid cells. Therefore, these cells undergo less of a shape change in order to complete the activation pathway.

The consequence of the spherical morphology of the treated platelets stored at 4°C has not been determined. The surface protein markers on these platelets indicate that these cells retain good expression of normal platelet plasma membrane proteins and little expression of activation markers (see the following data). We are currently examining the both the effects of this spherical morphology and possible mechanism to control the shape change in "in house" research programs.

The in vitro profile of stored platelets now include the following assays:

- 1) Cell number
- 2) ADP-induced aggregation
- 3) Collagen-induced aggregation
- 4) Hypotonic shock response
- 5) Extent of shape change
- 6) Transmission electron microscopy
- 7) FACS analysis of GPIb
- 8) FACS analysis of GMP-140 (CD62)

The analysis of all of these parameters presents an overall in vitro profile of the stored platelets. This measurement of the platelet characteristics represents a tool to define the ability of ThromboSol to protect platelets during storage at 4°C.

In Vitro Composite Analysis

To establish a complete composite picture of platelets stored at 4°C in the presence of

ThromboSol, functional activity data for a statistically significant number of control units is necessary. Toward this goal, multiple units of platelets were obtained and stored at 22°C according to conventional methods or at 4°C without ThromboSol, without shaking, in parallel to the methods applied to ThromboSol treated PC. The recovery of functional activity values for these units was determined over time and used for comparison to the data for ThromboSol treated PC stored a 4°C which has been accumulated over the course of this project. Table 2 displays the results of the comparison of 22°C stored control platelets, 4°C stored control platelets and 4°C stored ThromboSol-treated platelets.

Table 2

	Storage of Platelets for 1 Day					
	ThromboSol 4°C		22°C Control		4°C Control	
Assay	n	% +/- std dev. ¹	n	% +/- std dev.	n	% +/- std dev.
ADP Agg.	29	55 +/- 10	14	37 +/- 19	9	44 +/- 17
Collagen Agg.	13	90 +/- 6	10	81 +/- 9	4	94 +/- 6
HSR - 15 min	20	74 +/- 7	9	75 +/- 14	6	58 +/- 8
HSR - 4 min	16	61 +/- 15	4	67 +/- 7	5	46 +/- 11
ESC	16	13.6 +/- 3.5	4	23.2 +/- 5.0	5	11.9 +/- 3.4
GPIb	20	65 +/- 11	2	66 +/- 11	2	52 +/- 11
GPIb _{MFU} ²	20	4.2 +/- 0.7	2	6.5 +/- 0.1	2	4.9 +/- 0.6
GMP-140	20	41 +/- 11	2	48 +/- 18	2	45 +/- 5
GMP-140 _{MFU}	20	5.4 +/- 1.1	2	9.3 +/- 3.7	2	6.7 +/- 1.5

¹Mean values with the indicated n values.

²MFU is mean fluorescent units

	Storage of Platelets for 5 Days					
	ThromboSol 4°C		22°C Control		4°C Control	
Assay	n	% +/- std dev.	n	% +/- std dev.	n	% +/- std dev.
Cell # (% of 1)	20	92 +/- 8	11	89 +/- 7	8	59 +/- 29
ADP Agg.	20	45 +/- 8	12	16 +/- 7	9	25 +/- 6
Collagen Agg.	8	80 +/- 6	10	74 +/- 12	3	63 +/- 9

HSR - 15 min	10	61 +/- 11	12	69 +/- 17	6	60 +/- 20
HSR - 4 min	12	35 +/- 15	4	71 +/- 8	5	34 +/- 6
ESC	12	10.5 +/- 3.0	4	19.3 +/- 4.0	5	6.3 +/- 2.1
GPIb	15	74 +/- 9	2	66 +/- 11	2	61 +/- 15
GPIb _{MFU}	14	4.5 +/- 0.6	2	6.1 +/- 0.5	2	5.2 +/- 0.5
GMP-140	15	44 +/- 10	2	52 +/- 10	2	53 +/- 4
GMP-140 _{MFU}	15	5.6 +/- 1.2	2	8.5 +/- 2.8	2	6.7 +/- 2.1

	Storage of Platelets for 9 Days					
	ThromboSol 4°C		22°C Control		4°C Control	
Assay	n	% +/- std dev.	n	% +/- std dev.	n	% +/- std dev.
Cell # (% of 1)	15	91 +/- 12	6	87 +/- 5	8	57 +/- 23
ADP Agg.	13	43 +/- 6	7	13 +/- 8	7	15 +/- 7
Collagen Agg.	12	72 +/- 9	7	65 +/- 11	4	40 +/- 17
HSR - 15 min	12	48 +/- 8	7	74 +/- 15	5	48 +/- 18
HSR - 4 min	6	20 +/- 12		N.D.	5	25 +/- 4
ESC	3	11.6 +/- 2.3		N.D.	5	6.0 +/- 1.3
GPIb	12	64 +/- 7	1	64	2	67 +/- 12
GPIb _{MFU}	12	4.6 +/- 0.9	1	4.2	2	4.7 +/- 0.1
GMP-140	12	50 +/- 9	1	55	2	54 +/- 7
GMP-140 _{MFU}	11	5.9 +/- 1.0	1	6.0	2	6.9 +/- 1.8

The results of this comparison are as follows:

- 1) Treated platelets at day 1 display a strong recovery of agonist-induced aggregation which is significantly higher than the control platelets.
- 2) Treated platelets at day 1 show good HSR which is equivalent to 22°C conventional stored platelets.

- 3) Treated platelets at day 1 express low amounts of the activation marker CD62 (GMP-140).
- 4) Treated platelets at day 1 display about 60% of ESC as compared to conventional platelets.
- 5) In contrast to control platelets stored at 4°C, ThromboSol-treated platelets display >90% recovery of cell number.
- 6) Treated platelets retain the strong agonist-induced activation at both 5 days and 9 days. This activity is significantly higher than 22°C control platelets.
- 7) At day 5, treated platelets display equivalent recovery of HSR to control platelet at 22°C at 15 minutes, although the initial HSR rate is diminished.
- 8) Treated platelets at day 5 yield about 50% recovery of ESC as compared to conventional platelets, but almost 2 times the control platelets at 4°C.
- 9) All of the platelet samples had good expression of GPIb, while the treated sample shows both a lower percentage of cells expressing GMP-140 and a lower epitope to cell ratio.
- 10) At day 9 the treated platelets displayed good recovery of ESC and HSR equal to 60% and 70% of control platelets at 22°C at day 5, respectively.
- 11) At day 9 the treated cell continues to express GPIb on the surface and show little increase in the activation marker GMP-140.

Sterile ThromboSol

An important aspect of the use of ThromboSol in transfusion medicine is the ability to directly transfuse the formulation during infusion of the 4°C stored PC. In order to achieve direct transfusion, the original ThromboSol which is added to the PC to allow extended storage at 4°C must be sterile. Previously, ThromboSol was added to PC as a non-sterile suspension in buffered saline. To develop a method of application of ThromboSol which allowed for an intact, sterile PC, the ThromboSol suspension was autoclaved at 260°F for 30 min. at 29 lb/in² psig to achieve a sterile solution. The sterile ThromboSol was then tested for its ability to protect platelets from storage lesion during incubation at 4°C for 9 days. Table 3 demonstrates the functional recovery values for PC (n value = 5) stored at 4°C in the presence of sterile ThromboSol.

Table 3

Recovery of Platelets Stored at 4°C with Sterile ThromboSol (%)			
	Day 1	Day 5	Day 9
Cell Number (x10 ⁶ /ml)	1010	841	801
% ADP Aggregation ¹	57 +/- 9	51 +/- 12	44 +/- 9
% ESC	11.9 +/- 1.5	10.9 +/- 3.3	12.7 +/- 2.4
% HSR	66.4 +/- 8.1	37.7 +/- 14.6	26.1 +/- 5.9
GPIb ²	94.3	92.6	92.1
GMP-140 ²	41.6	49.3	54.8

¹Mean value +/- standard deviation.

²Percentage of the population expressing the indicated marker.

The functional activity values generated from the PC stored in the presence of autoclaved ThromboSol are equivalent to those previously achieved with non-sterilized ThromboSol treated platelets. This data demonstrates that autoclaved ThromboSol performs as well as the untreated formulation. Thus, by autoclaving the ThromboSol solution prior to the application the PC that the units for storage can be maintained in a sterile condition ready for direct and immediate transfusion.

Reduced Volume of Platelet Concentrates

An important issue in blood banking is the volume of plasma that is generated during the production of a PC for storage. Following the generation of a PRP fraction from the whole blood unit, the PRP is centrifuged and the plasma supernatant is separated from the PC. In order to achieve successful storage of the PC using conventional methods the PC must contain 60 ml of plasma. This plasma requirement in the PC yields a final plasma fraction which in many cases is of a lower volume than required for fresh frozen plasma (FFP). If the volume of plasma needed for successful storage of PC could be reduced, the number of FFP units obtained from whole blood units could be increased. The ability of ThromboSol to protect platelets from storage lesion may also allow for a reduction of the plasma volume needed in a PC to sustain recovery of functional activity. To determine the ability to store ThromboSol-treated PC at 4°C with a reduced plasma volume, PC were generated which contained only 30 ml total volume. Two PC were treated with ThromboSol, based on the 30 ml volume and two PC were treated with ThromboSol, based on a normal 60 ml volume (i.e. same ThromboSol/cell number ratio). These units were stored at 4°C and analyzed for recovery of functional activity over time. Table 4 shows the results of the ability of ThromboSol to protect platelets stored at 4°C as reduced volume PC (30 ml).

Table 4

Functional Activity of ThromboSol-Treated PC Stored with 30 ml of Plasma (mean values)						
	Cell Number (x10 ⁶ /ml)			% ADP Aggregation		
Time (days)	1	5	9	1	5	9
PC w/ 1x ThbSl	1002	1062	881	72.0	33.5	42.5
PC w/ 2x ThbSl	1087	992	960	50.5	34.0	35.5

	% ESC			% HSR		
Time (days)	1	5	9	1	5	9
PC w/ 1x ThbSl	9.9	11.0	7.3	43.7	16.0	0
PC w/ 2x ThbSl	11.2	15.4	8.7	56.2	24.9	16.0

This data demonstrates that the ThromboSol to platelet ratio is important for the ability of ThromboSol to protect against storage lesion during 4°C storage. In addition, the results show that storing PC in a reduced volume yields slightly lower recovery of platelet functionality as compared to a conventional 60 ml PC.

Inhibition of Cytokine Production

An important issue currently being addressed in transfusion medicine is the non-hemolytic febrile transfusion reaction. It has been demonstrated that this febrile reaction in patients following transfusion is the result of cytokines present in the PC. During storage at 22°C, the white blood cells (WBC) contaminants present in a PC produce various cytokines including IL1- β , TNF- α , and IL6, all of which can contribute to the induction of a febrile reaction in patients. The cytokines present in PC can be reduced by leukodepletion of the PC prior to storage in order to reduce the WBC contaminant cell number. The leukodepletion process, though, is both costly and labor intensive. In contrast to the conventional storage at 22°C, the ThromboSol treated PC are stored at 4°C, a temperature which may abrogate the metabolism of WBC, thus reducing the generation of the offending cytokines.

To address this question, PC were treated with ThromboSol and stored at 4°C as described previously in this report and these PC were analyzed for the presence of various cytokines over the storage period. Briefly, PC (n=5) were stored at 22°C without ThromboSol or at 4°C with ThromboSol for ten days. At the indicated time interval, an aliquot was harvested and the concentration of the cytokines IL6, TNF and IL1- β was determined by ELISA kits. The average WBC contamination for all of the units was 6.8×10^5 cells / ml. Table 5 indicates the generation of cytokines during storage of platelets under these two conditions.

Table 5

IL6 Production (pg/ml)				
Days	1	4	6	10
Control-22°C	2.5	2.8	7.8	12.6
Control-22°C	4.1	11.1	35.0	46.8
Control-22°C	5.7	272.3	215.8	203.7
Control-22°C	3.9	30.3	78.5	140.6
Control-22°C	2.2	179.9	278.0	262.4
ThromboSol-4°C	2.2	2.3	4.6	4.1
ThromboSol-4°C	4.0	3.2	2.4	2.4
ThromboSol-4°C	3.6	2.4	1.7	2.5
ThromboSol-4°C	4.2	2.6	2.5	2.5
ThromboSol-4°C	2.1	2.8	2.2	3.5

TNF Production (pg/ml)				
Days	1	4	6	10
Control -22°C	10.4	8.2	7.7	13.0
Control -22°C	7.6	17.9	22.5	26.3
Control -22°C	9.7	44.8	80.7	69.1
Control -22°C	9.4	17.5	23.1	27.8
Control -22°C	6.3	68.8	81.3	95.6
ThromboSol-4°C	6.5	3.8	3.4	4.4
ThromboSol-4°C	4.9	4.7	4.9	4.3
ThromboSol-4°C	4.7	5.7	13.6	3.4
ThromboSol-4°C	3.0	4.7	11.6	5.4
ThromboSol-4°C	8.1	3.9	4.7	7.3

	IL1- β Production (pg/ml)			
Days	1	4	6	10
Control -22°C	.54	.53	1.6	2.8
Control -22°C	.38	3.7	10.6	22.3
Control -22°C	.26	44.5	180.7	252.9
Control -22°C	.33	4.7	6.8	22.7
Control -22°C	.85	57.7	180.7	250.0
ThromboSol-4°C	.50	.53	.85	1.9
ThromboSol-4°C	.69	.80	.50	.69
ThromboSol-4°C	.63	.74	.69	1.2
ThromboSol-4°C	.69	.57	.45	.51
ThromboSol-4°C	.33	.45	.39	.22

The PC stored at 22°C produced high levels of all three cytokines. In contrast, the ThromboSol-treated PC stored at 4°C displayed no production of cytokines, regardless of the WBC number. These results indicate that storage at 4°C abrogates the production of cytokines by the WBC contaminants of PC. The obvious high level of cytokines generated during conventional 22°C storage is indicative of the advantage of achieving effective storage at 4°C.

As part of the control arm of this experiment, PC (n=5) were incubated at 22°C with ThromboSol or at 4°C without ThromboSol. As before at the indicated time point an aliquot of the PC was harvested and tested only for the concentration of IL6 by the ELISA assay.

Table 6

	IL6 Production (pg/ml)			
Days	1	4	6	10
ThromboSol-22°C	.05	0.0	.26	.71
ThromboSol-22°C	.47	.59	.52	.50
ThromboSol-22°C	.20	.86	.79	.42
ThromboSol-22°C	.20	.91	2.02	.99
ThromboSol-22°C	.13	.37	.42	.61

Control-4°C	.62	.91	1.64	1.10
Control-4°C	.05	.33	.56	.28
Control-4°C	.28	.29	0.0	.05
Control-4°C	.05	.42	.40	.24

As expected, 4°C storage alone was enough to inhibit the production of cytokines. Surprisingly, the 22°C stored PC with ThromboSol did not display any cytokine production. Thus it appears that the components of ThromboSol which affect the second messenger pathways in platelets may also exert a biochemical effect on the WBC component of PC during room temperature storage which inhibits the production of cytokines by these white blood cells.

The inhibitory effect of ThromboSol on WBC production of cytokines in 22°C stored PC was further verified by the following experiment. PC were obtained from the blood bank and split into two 30 ml aliquots in identical platelet storage bags. To one of the two split samples ThromboSol was added at the appropriate dose. Both samples, control and treated, were maintained at 22°C with agitation according to conventional methods. Alternatively, five units were split and treated similarly and placed at 4°C without shaking. At the indicated interval, an aliquot of the PC was tested for the generation of cytokines by ELISA kits.

Table 7

IL6 Production (pg/ml) - Storage at 22°C						
Sample	WBC (10 ⁵ /ml)	Treatment	Day 1	Day 3	Day 5	Day 10
1	7	CT	3.52	159.9	572.9	617.9
		ThromboSol	2.25	5.2	3.95	13.1
2	24	CT	2.32	127.2	571.2	605.6
		ThromboSol	2.80	15.4	65.6	66.9
3	14	CT	2.15	13.3	118.1	295.5
		ThromboSol	3.08	18.9	34.6	31.9
4	16	CT	2.39	125.6	382.2	342.9
		ThromboSol	2.66	41.0	142.7	149.7
5	8	CT	2.45	3.06	35.6	44.6
		ThromboSol	2.46	3.37	4.38	3.99

IL1- β Production (pg/ml) - Storage at 22°C						
Sample	WBC (10 ⁵ /ml)	Treatment	Day 1	Day 3	Day 5	Day 10
1	7	CT	0	5.12	83.08	160.33
		ThromboSol	0	0.92	.74	2.86
2	24	CT	7.86	29.46	187.16	311.70
		ThromboSol	7.84	11.21	29.50	41.30
3	14	CT	13.47	17.00	29.27	99.39
		ThromboSol	13.17	14.56	20.08	27.6
4	16	CT	.460	11.40	177.8	239.2
		ThromboSol	.092	2.43	13.0	28.28
5	8	CT	0	0	4.65	16.6
		ThromboSol	0	.408	1.63	.63

IL6 Production (pg/ml) - Storage at 4°C						
Sample	WBC (10 ⁵ /ml)	Treatment	Day 1	Day 3	Day 5	Day 10
1	18	CT	2.26	2.39	1.57	1.74
		ThromboSol	2.53	2.25	2.08	1.82
2	11	CT	2.65	2.39	2.76	1.45
		ThromboSol	2.52	2.66	1.99	2.15
3	15	CT	2.73	2.87	2.32	2.25
		ThromboSol	2.87	2.59	2.33	2.32
4	11	CT	3.38	3.01	2.15	1.81
		ThromboSol	2.53	1.73	1.91	1.32
5	11	CT	3.15	2.13	2.51	0
		ThromboSol	2.94	0.63	2.69	0

As a control for this experimental series the cellular cytokine content was examined in the PC stored under the above conditions to rule out the possibility that the WBC in the treated unit were

generating cytokines but not releasing them into the plasma. Cell samples from the PC analyzed above were isolated by centrifugation and lysed by detergent treatment. The resulting cell lysis mixture was then analyzed for the total cellular content of the representative cytokine IL6. The data in Table 8 demonstrates that in the ThromboSol-treated PC at 22°C and all of the 4°C stored units no amount of cytokines were produced intra-cellularly. In contrast, as seen in the plasma determination of cytokine concentration, the 22°C stored control PC displayed high levels of cytokines in the cellular fraction.

Table 8

IL6 (WBC Cellular Content in pg/ml)			
		IL6 Production	
Sample	temperature	Control	Treated
1	22	87.6	0
2	22	96.14	18.9
3	22	34.16	3.47
4	22	59.17	17.24
5	22	ND*	ND
1	4	ND	ND
2	4	ND	ND
3	4	ND	ND
4	4	ND	ND
5	4	ND	ND

* None detected

This data confirms the previous results by demonstrating that the treatment of PC with ThromboSol prevents the generation of cytokines during conventional storage at 22°C. In addition, the storage of PC at 4°C also inhibits cytokine production. Storage of platelets at 4°C with recovery of cell number and functional activity can be achieved by the inclusion of ThromboSol.

Storage of Leukodepleted PC with ThromboSol

It is becoming increasingly common in the field of platelet transfusion to employ leukodepletion

filters to remove white blood cells (WBC) during the harvesting of PC. There are two major reasons for the implementation of the leukodepletion step. First is the host-versus-graft immunological problems associated with the HLA antigens on the transfused WBC. The second reason is the WBC-produced cytokines during standard 22°C storage, which are responsible for a febrile response from the recipient. The removal of the WBC prior to storage alleviates these effects. While the previous results have demonstrated the ability to reduce cytokine production by the inclusion of ThromboSol, this would not alleviate the HLA associated problems. Since leukodepletion represents a processing step which can be expected to become an integral component of PC production, platelet storage experiments were done to examine the functional activity recovery of leukodepleted PC stored at 4°C with ThromboSol. The experiments were performed as follows:

- 1) PRP units were obtained from the Gulf Coast Regional Blood Center
- 2) The PRP units were leukofiltered by gravity filtration using a Miles/Cutter leukodepletion system.
- 3) The leukodepleted PRP was then processed according normal blood banking protocols to yield a PC.
- 4) The PC was treated with ThromboSol, stored at 4°C, and analyzed for functional activity recovery as described previously.

Table 9 displays the recovery of functional activity of leukodepleted PC stored at 4°C with the addition of ThromboSol.

Table 9

Analysis of ThromboSol in Leukodepleted PC Stored at 4°C						
Assay	Day 1		Day 5		Day 9	
	n	mean +/-std dev	n	mean +/-std dev	n	mean +/-std dev
Cell number ¹	10	867	10	879	6	805
% ADP Aggregation	10	34 +/- 16	10	28 +/- 17	6	31 +/- 14
% ESC	10	11.6 +/- 1.8	10	8.5 +/- 1.2	6	8.2 +/-1.6
% HSR - 4 min	10	54.5 +/- 8.0	10	40.6 +/- 4.0	6	26.9 +/- 8.2
% HSR - 15 min	10	78.8 +/- 10.0	10	68.7 +/- 8.0	6	52.0 +/- 8.4
GPIb ²	10	93.7 +/- 3.7	10	94.0 +/- 2.0	10	92.8 +/- 2.9

GPIb _{mfu}	10	24.6 +/- 4.5	10	28.7 +/- 5.9	10	31.7 +/- 3.0
GMP-140 ²	10	37.9 +/- 19.3	10	37.0 +/- 17.0	10	33.4 +/- 11.5
GMP-140 _{mfu}	10	5.1 +/- 1.3	10	4.7 +/- 0.9	10	4.4 +/- 0.5

¹ Cell number (x 10⁶/ml)

²Percent of platelet population expressing the indicated marker.

The results of this experimental series demonstrate the leukodepletion of PC has no effect on the ability of ThromboSol to protect platelets against storage lesion during incubation at 4°C.

Cryopreservation of Platelets with ThromboSol

In the previous report we demonstrated that the ThromboSol formulation effectively protects platelets during cryopreservation. In these experiments the platelet population, following the freeze-thaw process, yielded good recovery of cell number and functional activity. In addition, it was demonstrated that the effective concentration of DMSO, which gives this good recovery, could be reduced from the conventional level of 6% to a final concentration of 2%. Furthermore, the cryopreservation procedure for the platelets was optimized to allow for ease of processing of the cells. The previous cryopreservation experiments were performed using the original concentration of ThromboSol reagents (see the year two report). The data presented in the year two report demonstrated that a reformulation of the concentration of the ThromboSol reagents yielded equivalent protection of platelets stored at 4°C. As outlined in that report, this reformulation allowed for the reduction of several of the components of ThromboSol. The new formulation which is described in the Background Section of this report was tested for its ability to protect platelets during cryopreservation. This protocol is described below.

Preparation of platelets:

- Platelet concentrates (PC) were obtained from the Gulf Coast Regional Blood Bank.
- The PC were held at 22°C overnight for biohazard testing. In some preliminary experiments the units were treated immediately followed by cryopreservation. These units displayed identical results to the "held" units and thus the subsequent experiments used held units so that PRP values could be measured.
- The PC were treated with appropriate amounts of ThromboSol with gentle massaging to cultivate mixing.
- DMSO was added directly to the PC to achieve 2% final concentration.
- The PC were put into a blood freezer cassette and placed directly into a -80°C freezer.
- At the time of thaw, the PC were removed from the -80°C freezer and placed into a 37°C water bath until completely thawed.
- The platelets were pelleted at 900 x g for 20 minutes followed by resuspension in autologous PPP to a cell count of 300 x 10⁶ cell/ml.
- Recovery assays were performed on these samples to determine in vitro functional activity.

The advantages of this method are two-fold. First, the PC are not diluted as with the Valeri method, since the DMSO is added directly. Secondly, the DMSO is added as a bolus with no laborious methods of addition as with the conventional method.

PC were prepared and cryopreserved as described above and analyzed for recovery of in vitro functional activity according to the following parameters:

- 1) Swirling Index: The platelets were tested for the amount of "swirl" as an indication of the percentage of the platelet population which are discoid in morphology. The swirl scale is designed on a 0-4 basis, with 4 equal to 100% discoid and 0 equal to 100% spherical.
- 2) Cell Number
- 3) ADP-Induced Aggregation
- 4) Hypotonic Shock Response
- 5) Extent of Shape Change

Table 10 displays the results of the application of ThromboSol with 2% DMSO to unit level preparations of platelets as compared to control platelets prepared according to standard AABB protocols.

Table 10

Cryopreservation of ThromboSol-Treated Platelet Units						
	Control Plt ¹ w/ 6% DMSO		Control Plt ² w/ 2% DMSO		Treated Plt ³ w/ 2% DMSO	
	absolute value*	% of fresh PRP	absolute value	% of fresh PRP	absolute value	% of fresh PRP
Cell Number ⁴	922	66	834	60	1305	94
Swirl Index	2	50	0	0	3	75
%ADP Aggregation	4	19	8	38	14	67
% ESC	14.3	48	14.6	49	16.6	57
% HSR	41	65	39	61	48	76

¹Control platelets prepared by the conventional 6% DMSO dilution technique.

²Control platelets with the direct addition of 2% DMSO.

³ThromboSol-treated platelets with direct addition of 2% DMSO.

⁴Cell number times 10⁶/ml

*Mean percentage value with a n=5.

The results of this experimental series are as follows:

1) The addition of ThromboSol during cryopreservation yields a high recovery of cell number (>90% of fresh PRP). In contrast, both control methods yielded a significant loss of platelets (>40%). This low recovery of cell number with the standard AABB cryopreservation method has been observed in previously published work.

2) The treated platelets maintained good discoid morphology following freeze-thaw. These platelets displayed a swirl index of 3 indicating a large percentage of the platelet population retained their discoid shape. The control platelets showed a diminished discoid population.

3) The ThromboSol-treated platelets cryopreserved with 2% DMSO displayed good recovery of functional activities. The critical in vitro measurements of the treated platelets yielded a good activity profile for all indices, all of which were significantly higher than the control 6% DMSO treated platelets.

4) As compared to the fresh PRP, the platelets cryopreserved with ThromboSol and 2% DMSO retained functional activities (57% - 76%) indicating very little freeze-induced damage with this technique.

5) The increase in recovery of cell number in conjunction with the higher activity profile of the treated cells demonstrated that the addition of ThromboSol to platelets during cryopreservation allow for the extended storage of platelets which retain properties compatible with good circulatory retention.

In summary, these experiments to develop ThromboSol have demonstrated that the modulation of second messenger pathways can protect platelets against storage induced damage that leads to loss of cell number and activity. Moreover, this ThromboSol-induced biochemical protection of platelets can be extended to storage of platelets via cryopreservation. The application of ThromboSol to the cryopreservation of platelets allows for the simplification of the freezing techniques. This includes the direct addition of the cryoprotectant DMSO, non-controlled slow rate freezing, elimination of the dilution of the platelet units and storage at -80°C. Furthermore, the use of ThromboSol to protect platelets allows for the decrease of the DMSO concentration 3-fold from 6% to 2% while increasing the post-thaw yield with regards to cell number and function.

Conclusion

The overall conclusions from this project to develop a system to extend the storage of platelets are as follows:

1) The addition of second messenger effectors (ThromboSol) to modulate specific biochemical

pathways of a platelet prevents storage lesion at 4°C.

2) Following 9 days of storage at 4°C with ThromboSol storage solution, the platelets retain strong agonist-induced aggregation response indicating the potential for good hemostatic activity.

3) Treated platelets display recovery of the functional measurements of HSR and ESC comparable to published values shown to be consistent with satisfactory in vivo recovery and half-life in circulation.

4) The ThromboSol-treated platelets display no loss of expression of the surface marker GPIb and also show a diminished expression of the activation marker GMP-140 as compared to control platelets.

5) The ThromboSol storage solution-treated platelets demonstrate in vitro activity profiles compatible with survival in the circulation.

6) Since the application of ThromboSol storage solution to platelet concentrates overcomes the detrimental effects of cold storage, these cells can be stored for extended periods (9 days).

7) Storage of platelets with ThromboSol at 4°C lowers WBC cytokine generation.

8) The addition of ThromboSol solution to conventionally stored PC lowers cytokine production.

9) This ability to store platelet concentrates at 4°C, a temperature which is non-permissive for bacterial growth, should reduce the risk of transfusion-related sepsis in recipient patients and alleviate the requirement to outdate units at 5 days.

10) The addition of ThromboSol protects platelets against freeze-thaw damage during cryopreservation.

11) The application of ThromboSol to the cryopreservation system allows for the reduction of the DMSO concentration to 2% with no loss of recovery of functional activity.

The future goals of LifeCell's program to develop ThromboSol as system to extend storage of platelets for combat casualty care as follows:

A. Determine the optimal sterilization method, packaging and stability of the ThromboSol formulation and identifying any effects on platelet bag material. Optimize delivery of the formulation to platelets concentrates.

B. Demonstrate the capability to manufacture and deliver ThromboSol at a blood banking level.

C. Conduct pre-clinical studies to determine animal safety and preliminary pharmacology of ThromboSol formulation.

D. Demonstrate in vivo function in a rabbit hemostasis model system.

E. Prepare and file IND for testing safety and efficacy of ThromboSol-stored platelets.

F. Demonstrate the ability to apply ThromboSol to the cryopreservation of platelet units for long-term storage of platelets for stock-piling purposes.

G. Perform developmental experiments to define the parameters for the effective application of ThromboSol to the freeze-drying of platelets.

We are actively pursuing a corporate partner and further DOD funding to complete this program and realize the benefits of the ThromboSol product in the arena of combat casualty care.

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